Bile Salt-Modulated Stereoselection in the Cholesterol Esterase-Catalyzed Hydrolysis of α-Tocopheryl Acetates[1]

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Abstract: The noncompetitive and competitive hydrolyses of (2*R*, 4'*R*, 8'*R*)-α-tocopheryl acetate (*RRR*-α-TOAc, the acetate of natural vitamin E) and (2*S*,4'*R*,8'*R*)-α-tocopheryl acetate (*SRR*-α-TOAc) catalyzed by crude and pure bovine cholesterol esterase (BCE) and crude and pure porcine cholesterol esterase (PCE) have been studied at 37 °C. These two CE's are catalytically active toward tocopheryl acetates only in the presence of bile salts. The three 3α,7α,12α-trihydroxy bile salts, cholate, glycocholate and taurocholate, not only are effective activators of BCE and PCE, but also modulate the diastereoselectivities of these two enzymes in their hydrolyses of *RRR*- and *SRR*-α-TOAc in a characteristic manner. Rates of hydrolyses were much faster in the presence of a small quantity of *dl*- or *l*-dimyristoylphosphatidylcholine (DMPC) than in its absence. However, for each enzyme, the direction and even the magnitude of the diastereoselectivity is primarily determined by the bile salt employed and not by the presence or nature of the co-lipid (DMPC or sodium oleate), nor by the bile salt/co-lipid ratio, nor by the purity of the enzyme. In noncompetitive experiments, the ratios of the BCE-catalyzed initial rates of hydrolyses of the diastereomeric acetates, V_i^{RRR}/V_i^{SRR}, are 0.21, 1.5, and 2.7 for cholate, glycocholate, and taurocholate, respectively, and for the PCE-catalyzed noncompetitive reactions, 0.21, 7.9, and 7.5 for the same three bile salts. In competitive experiments using equal concentrations of *RRR*- and *SRR*-α-TOAc, the BCE-catalyzed initial rate ratios are 0.33, 0.94, and 2.2, and for the PCE-catalyzed competitive reactions, 0.21, 1.1, and 1.8 for cholate, glycocholate, and taurocholate, respectively. The lower diastereoselectivities found in some of the competitive experiments are tentatively attributed to competitive inhibition of the enzyme by one of the diastereomeric acetates or, more probably, its phenolic product. Glycochenodeoxycholate, a 3α,7α-dihydroxy bile salt, activates PCE but not BCE. With the former enzyme, the diastereoselectivities, V_i^{RRR}/V_i^{SRR} are rather similar for the noncompetitive and competitive experiments, viz., 11 and 8.0, respectively, and the reaction rates are comparable to those found with the trihydroxy bile salts. Since the detailed composition of the bile salt/co-lipid mixed micelle does not determine V_i^{RRR}/V_i^{SRR} ratios, we conclude that diastereoselectivities are not determined by the precise surface structure of the micelle. Presumably, the bile salts modulate the diastereoselectivities of BCE and PCE by a direct effect on the protein which may involve "refolding" the enzyme with a consequent change in the shape of the active site. It should be noted that the relevant chiral carbon in α-TOAc is separated by six bonds from the bond which is broken and that this chiral center must exert its influence prior to the rate-limiting step in the overall hydrolysis. If the bile salt modulating effect on CE-catalyzed reactions is to be exploited in organic syntheses, it is the competitive experiments which are the more important, and for these, the diastereoselectivity, V_i^{RRR}/V_i^{SRR}, varies from a low of 0.21 for the cholate/PCE couple to a high of 8.0 for the glycochenodeoxycholate/PCE couple. Such a dramatic 40-fold change in an enzyme's chiral selectivity is without precedent.

Introduction

Natural vitamin E, (2*R*,4'*R*,8'*R*)-α-tocopherol (*RRR*-α-TOH), and synthetic vitamin E, (2*RS*,4'*RS*,8'*RS*)-α-tocopherol (*all-rac*-α-TOH, an approximately equal mixture of the eight possible stereoisomers),[3] are generally sold to the consumer as esters, most usually as the acetate (see Chart 1). Since the intestinal absorption of α-tocopheryl acetate (α-TOAc) does not occur, the acetate must first be hydrolyzed to the free α-tocopherol which can then be absorbed. This hydrolysis occurs in the small intestine under the catalytic action of pancreatic juice enzyme(s). The enzyme which appears to be mainly responsible for the hydrolysis has commonly been referred to as cholesterol esterase (carboxyl ester hydrolase, EC 3.1.1.13). However, it has long been known that cholesterol esterase is, for all essential purposes, catalytically active only in the presence of bile salts, from which fact arises its modern name: bile salt-activated lipase or BAL[4] (for a review of the nomenclature and properties of BAL, see ref 4). We will continue to use the name cholesterol esterase or CE because it is less cumbersome than BAL particularly in the context of the present paper which deals with the bile salt-modulated diastereoselectivity of hydrolytic reactions catalyzed by this enzyme.

The obligatory role of bile salts on the rat[5–8] and human[7,9–11] CE-catalyzed hydrolysis of α-TOAc[5,7–10] and α-tocopheryl nicotinate[6] and esterification of α-TOH[11] has been demonstrated.

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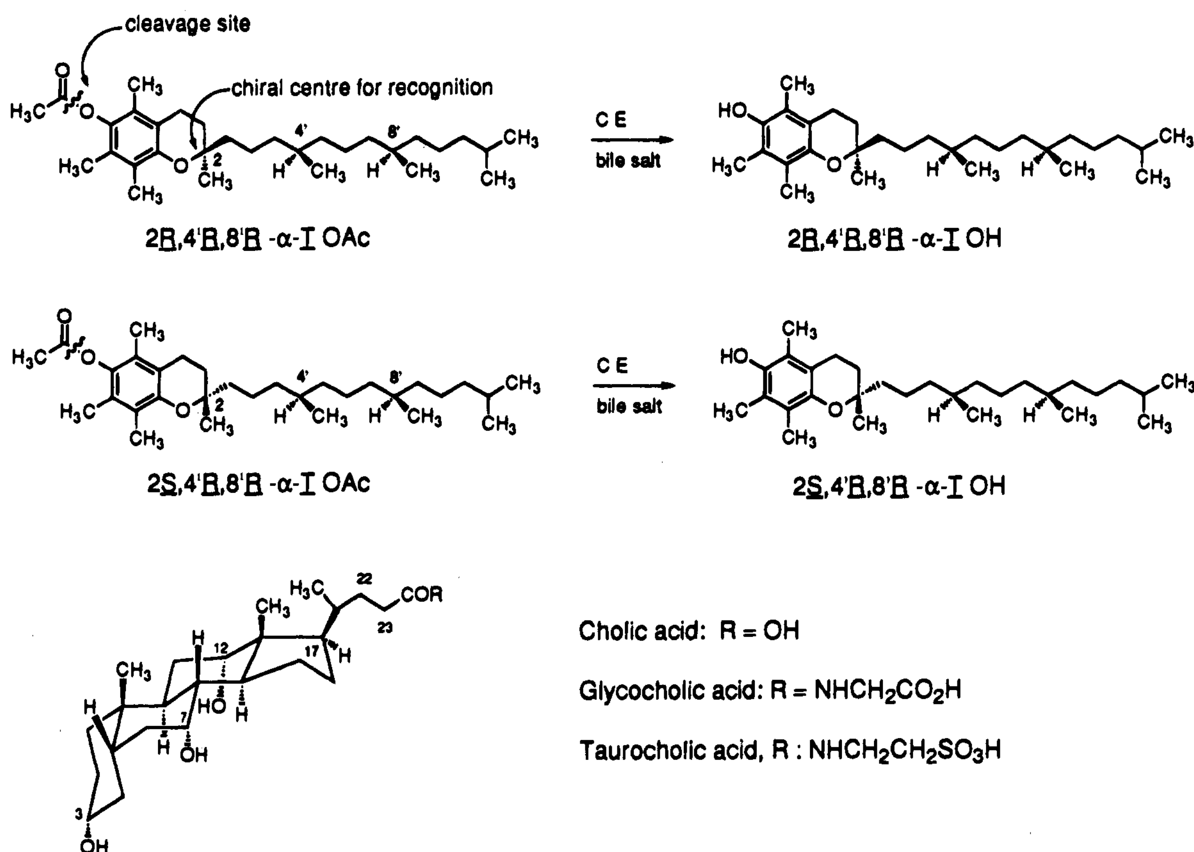
Chart 1



There is also much related work on other water-insoluble substrates.[7,10-19] The natural 3α,7α,12α-trihydroxy bile salts, cholate, glycocholate, and taurocholate (see Chart 1), are particularly effective activators of CE.[10,12-15,19-21] It would appear from the literature that the bile salts serve four functions:

(i) To activate CE.[5-20]

(ii) To function as detergents,[10,13-15,17,22-25] by forming micelles[22,23] and mixed micelles with (dietary) lipids[22,23,27,28] together with the ester substrate(s) which make(s) the ester(s) more readily available for the CE-catalyzed reaction.[7,8] This role also accounts for their stimulating effect in humans and in rats on the absorption of doses of the free phenols α-TOH[29] and γ-tocopherol,[30] respectively.

(iii) To protect CE from degradation by pancreatic proteases.[13-15,20] This protection apparently arises from a bile salt-induced oligomerization of the CE,[10,15,20,31] which produces the active form of the enzyme[31] which is more stable than the monomer.[31] (Deoligomerization to reform monomer occurs if the bile salt is removed.)[20]

(iv) To protect CE from denaturation at lipid−water interfaces,[32] probably by preventing a lipid-surface-induced conformational change which leads to deactivation.[32]

We have uncovered a fifth role for the bile salts: an unprecedented modulation of an enzyme's chiral selectively.[33,34]

In an early study[35] we demonstrated that the de-esterification of *RRR*-α-TOAc and of an "unnatural" stereoisomer, *SRR*-α-TOAc, could be catalyzed *in vitro* by bovine cholesterol esterase

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(BCE) when the substrates were dispersed in 40 mM sodium cholate/2 mM *dl*- or *l*-dimyristoylphosphatidylcholine (*dl*- or *l*-DMPC) mixed micelles at 37 °C in Tris buffer (pH 7.7). The results were analyzed by conventional Michaelis–Menten kinetics which indicated that $V_{max(RRR)}/V_{max(SRR)} \approx 1/6.8$. We subsequently recognized[33] that a Michaelis–Menten approach was invalid since it did not take into account the fact that CE was catalytically active only at (on) the surface of a micelle. As a consequence, the Michaelis–Menten kinetic terms, K_m and V_{max}, have no intrinsic meaning and are, in fact, dependent on the precise conditions of the kinetic experiments.[36,37] Nevertheless, despite our improper kinetic analysis, these early results[35] showed quite clearly that (pure) *RRR*-α-TOAc was hydrolyzed more slowly than (pure) *SRR*-α-TOAc by the BCE/sodium cholate/DMPC mixed micellar system. Significantly, the main result of these noncompetitive (single substrate) experiments was fully confirmed by an experiment in which an unlabeled *RRR*-α-TOAc-d_0 and an equal concentration of a trideutero-labeled *SRR*-α-TOAc-d_3 were hydrolyzed *competitively* by the BCE/cholate/DMPC micellar system and the rates of formation of *RRR*-α-TOH-d_0 and *SRR*-α-TOH-d_3 were measured by gas chromatography/mass spectrometry (GC/MS).[35] Under these competitive conditions also, *SRR*-α-TOAc was hydrolyzed more rapidly than *RRR*-α-TOAc. However, these *in vitro* results were found to be inconsistent with the results of some experiments carried out using rats which clearly demonstrated that, under *in vivo* conditions, the acetate of the natural stereoisomer of α-tocopherol (i.e., *RRR*-α-TOAc) was hydrolyzed *more* extensively and, presumably, more rapidly than the acetate of the "unnatural" stereoisomer (i.e., *SRR*-α-TOAc).[38,39]

The discrepancy between our *in vitro* and *in vivo* results prompted us to undertake a series of experiments carefully designed to discover the reason(s) for the difference. Competitive kinetic studies on the hydrolysis of *RRR*-α-TOAc-d_0 and *SRR*-α-TOAc-d_3 by a mixture of rat bile and rat pancreatic juice *and* by a mixture of rat bile and BCE conclusively demonstrated that the *in vitro/in vivo* difference in chiral selectivity was due to the composition of the rat's bile salts and not to any intrinsic differences in the diastereoselectivities for (α-TOAc) ester hydrolyses between CE derived from rat pancreas and CE derived from bovine pancreas.[33]

Although cholic acid is by far the major trihydroxy acid present in rat bile it is not present in detectable amounts as the free acid but rather as its taurine and glycine conjugates.[40–42] We therefore carried out both noncompetitive and competitive kinetic experiments involving the BCE-catalyzed hydrolyses of *RRR*-α-TOAc and *SRR*-α-TOAc in *l*-DMPC mixed micelles at 37 °C under conditions generally similar to those previously employed[35] using the sodium salts of cholic, glycocholic, and taurocholic acids. The results of these preliminary experiments[33] demonstrated that the diastereoselectivity of the BCE-catalyzed hydrolyses of α-TOAc's could be "modulated" by the bile salt used to "activate" the enzyme.

During our earlier studies,[33] we were kindly informed by Prof. D. M. Quinn that commercially available BCE preparations (which we had been using) were "extremely" impure. Because the potential role of these impurities in the observed chiral selectivity for α-TOAc hydrolysis was unknown, we set out to repeat *all* the experiments which had been done with crude, commercial BCE (cBCE) with the pure bovine enzyme (pBCE). For comparison, we also used crude, commercial porcine cholesterol esterase (cPCE) and the pure enzyme (pPCE). These two enzymes were prepared in pure form by affinity chromatography using sepharose bound cholate as the ligand.[43]

In the present paper, we give full details regarding both the absolute and the relative rates of hydrolysis of *RRR*-α-TOAc and *SRR*-α-TOAc in noncompetitive and in competitive experiments with the four enzyme preparations mentioned above in the presence of the three natural 3α,7α,12α-trihydroxy bile salts (Chart 1). Concentrations of these bile salts have been varied. The concentrations of the lipid (*l*-DMPC, *dl*-DMPC, and sodium oleate) which interacts with the bile salt to form the mixed micelles have also been varied. Some results for the CE-catalyzed hydrolysis of *RRR*-α-TOAc and *SRR*-α-TOAc modulated by a number of dihydroxy bile salts are also presented.

Our main findings are that the level of purity and the origin (i.e., cow or pig) of the CE has a much lesser influence on the degree of chiral discrimination of the enzyme (as measured by the relative rates of hydrolysis of *RRR*-α-TOAc and *SRR*-α-TOAc in competitive experiments) than does the structure of the trihydroxy bile salt used to activate the enzyme; however, the origin of the enzyme is very important in the case of the only dihydroxy bile salt exhibiting a strong activating effect on a CE.[34] The modulating effect of the bile salts on the CE's chiral selectivity is truly remarkable in that the *bond cleaved by the esterase is six bonds removed from the chiral center* (see Chart 1).

Experimental Section

Materials. The syntheses of *SRR*-α-TOAc-d_3, *RRR*-α-TOAc-d_6, and *all-rac*-α-TOH-d_9 have been described previously.[44,45] Crude cholesterol esterase (EC 3.1.1.13) from bovine pancreas (Genzyme #70-1081-

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(38) Analyses of the fecal material from the large intestine of nine rats which had been continuously fed for up to 65 days with a diet containing an equimolar mixture of hexadeutero-labeled *RRR*-α-TOAc-d_6 and trideutero-labeled *SRR*-α-TOAc-d_3 yielded a mean ratio of [*RRR*-α-TOAc-d_6]/[*SRR*-α-TOAc-d_3] = 0.37 and a mean ratio of [*RRR*-α-TOH-d_6]/[*SRR*-α-TOH-d_3] = 1.36. See Table 6 in the following: Ingold, K. U.; Burton, G. W.; Foster, D. O.; Hughes, L.; Lindsay, D. A.; Webb, A. *Lipids* **1987**, *22*, 163–172.

(39) An equimolar mixture of *RRR*-α-TOAc-d_6 and *SRR*-α-TOAc-d_3 (combined dose, 6 mg/kg of body weight) dissolved in tocopherol-stripped corn oil was administered to rats which had been fasted for 24 h, prior to which they had been maintained since weaning at 3 weeks for 55 days on a vitamin E-sufficient diet and the contents of the forward (i.e., closest to the stomach), middle, and final third of the small intestine were analyzed. In the forward third the [*RRR*-α-TOAc-d_6]/[*SRR*-α-TOAc-d_3] and [*RRR*-α-TOH-d_6]/[*SRR*-α-TOH-d_3] ratios were 0.90 and 1.08 at 1 h, 0.99 and 1.01 at 2 h, α-TOAc's too low to measure and 1.16 at 4 h, and ditto and 1.38 at 8 h, respectively. Similar data for the middle third were 0.68 and 1.22 at 1 h, 0.80 and 1.28 at 2 h, 0.58 and 1.28 at 4 h, and α-TOAc's too low to measure and 1.27 at 8 h, for the final third, data were ~1 and 1.43 at 1 h, 0.73 and 2.34 at 2 h, 0.40 and 1.53 at 4 h, and 0.46 and 1.27 at 8 h. Control experiments in which the rats were dosed with an equimolar mixture of *RRR*-α-TOH-d_6 and *SRR*-α-TOH-d_3 showed equal amounts of these two compounds present at all times in all three segments of the small intestine. This proves that the results obtained with the acetates in the single dose experiments (which demonstrate preferential hydrolyses of *RRR*-α-TOAc) are not influenced by any differences in the speed of absorption of the two free phenols in the intestine.

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01, 15 units/mg, powder: cBCE) and from porcine pancreas (Sigma C-9530, lyophilized powder, 70% protein: cPCE) was purified as described in the following subsection.

Sodium cholate, glycocholate, taurocholate, taurodeoxycholate, taurochenodeoxycholate, taurolithocholate, and glycochenodeoxycholate were purchased from Sigma. Sodium glycocholate, deoxycholate, chenodeoxycholate, ursodeoxycholate, glycoursodeoxycholate, tauroursodeoxycholate, and taurolithocholate were purchased from Calbiochem. In our initial experiments[33] the three trihydroxy bile salts were used "as received". A change in the source from which glycocholate was purchased produced a rather dramatic change in the chiral discrimination for hydrolyses of *RRR*- and *SRR*-α-TOAc with various CE preparations modulated by this bile salt. The three 3α,7α,-12α-trihydroxy bile salts and three 3α,7α-dihydroxy bile salts were therefore recrystallized either as the free acid (cholic acid from 95% EtOH) or as the sodium salts (glycocholate and taurocholate from 95% EtOH/Et_2O, chenodeoxycholate from *i*-PrOH/MeOH/H_2O (5:2:1), glycochenodeoxycholate from *i*-PrOH/H_2O (8:1), and taurochenodeoxycholate from EtOH/H_2O (5:1)), and their purity was checked by ^{13}C NMR.

The following materials were obtained from the sources indicated: disodium EDTA (Fisher), *l*-dimyristoylphosphatidylcholine (Avanti Polar-Lipids Inc.: *l*-DMPC), *dl*-DMPC (Fluka), Tris buffer (a mixture of Tris(hydroxymethyl)aminomethane and its hydrochloride which was made up to pH 8.3 at 25 °C, corresponding to a pH of 8.0 at 37 °C, was used throughout most of this work) and sodium oleate (Sigma), sephacryl S-200-HR, sepharose CL-6B, and gel-filtration molecular weight standards (Pharmacia Fine Chemicals), sodium chloride (AnalaR, BDH), HPLC grade organic solvents (Aldrich or BDH). Distilled, deionized water suitable for work with enzymes was employed in the preparation of all solutions.

Enzyme Purification. During chromatographic purification, the protein concentrations in individual column fractions were estimated spectrophotometrically from the absorbances at 260 and 280 nm. In the final activity assays the protein concentrations were measured by the Lowry method.[46,47] Electrophoresis was performed on 15% SDS polyacrylamide gels according to the procedure of Laemmli.[48] Molecular weight estimations were carried out on a Sephacryl S-200-HR column (16 mm × 30 cm) equilibrated with 50 mM Tris buffer (pH 8.0) and 1 mM EDTA and on a Sepharose CL-6B column (25 mm × 55 cm) under the same conditions. Blue dextran was used to determine the void volumes of these columns. Pharmacia molecular weight markers were used to calibrate the columns. Purifications and molecular weight determinations were carried out at 4 °C unless otherwise noted.

Crude bovine and porcine cholesterol esterases were purified by affinity column chromatography using a modification of Wang's procedure.[43] Details of the purification will be reported elsewhere. Fractions eluting from the column that showed esterase activity were evaluated for purity by SDS-PAGE (silver staining). Those judged pure were pooled and concentrated by ultrafiltration. These are the preparations referred to as pBCE and pPCE.

(Approximate) Standardization of the α-TOAc Hydrolyzing Activity of CE Preparations. Aqueous CE solutions were thawed and maintained at 0 °C on wet ice. A 1.5 mL solution containing 100 μM *SRR*-α-TOAc-d_3 in sodium cholate (40 mM)/*l*-DMPC (2.0 mM) micelles was prepared as described in Preparation of Standard α-TOAc/*l*-DMPC Mixtures. Hydrolysis at 37 °C was initiated with a volume of the CE solution sufficient to produce an initial rate of formation of *SRR*-α-TOH-d_3 of about 0.05 $\mu M\ s^{-1}$ (i.e., 5.0×10^{-8} M s^{-1}). In subsequent experiments, the same relative volume of the CE solution was added to each sample for which rate measurements were to be made.

In these "standardization" experiments, the rate of formation of *SRR*-α-TOH-d_3 was, for reasons of speed and "simplicity", monitored spectrophotometrically using the change in the absorbance at 302 nm [$\epsilon_{\alpha\text{-TOH}} - \epsilon_{\alpha\text{-TOAc}} = 2300$ L mol^{-1} cm^{-1})[33,35] as in some of our earlier work.[33,35] Unfortunately, there are problems with this "simple" spectroscopic kinetic technique. For our "proper" kinetic experiments (which constitute all the rate data tabulated in this paper unless otherwise noted), we employed the much more reliable procedure of chemical analysis by GC/MS for the α-TOH formed and α-TOAc lost as a function of time. This procedure demonstrated that the rates of formation of *SRR*-α-TOH-d_3 in the cholate-modulated, CE-catalyzed hydrolysis of the acetate under the "standardized" conditions were not 5.0×10^{-8} M s^{-1} in each case but varied from 7×10^{-8} to 11×10^{-8} M s^{-1} (see the Results). Thus, the *SRR*-α-TOAc hydrolytic activities of the four CE preparations employed in our kinetic studies were somewhat less "standardized" than we had intended. Fortunately, the fact that the four CE preparations did not hydrolyze *SRR*-α-TOAc at exactly equal rates under the influence of cholate has no impact on the major thrust of this paper (which is on the effect of different bile salts on the *relative* rates of the CE-catalyzed hydrolysis of *RRR*- and *SRR*-α-TOAc).

Enzyme Molecular Weight. The reduction of pPCE and pBCE was achieved by boiling in a solution containing SDS, mercaptoethanol, and glycerol for 3 min. The molecular weights of the reduced pPCE and pBCE were estimated to be 58 kDa and 65 kDa, respectively, by SDS PAGE.

Collection of Rat Bile and Pancreatic Juice. Male Sprague–Dawley rats were raised and maintained on an AIN-76A diet[49] at the NRCC's pathogen-free facility. Bile and pancreatic juice free from bile were collected from a halothane anesthetized rat following a published procedure.[14] The animal recovered in a restraining cage, and the collection of bile and pancreatic juice continued for 4 h. The entire bile sample was refrigerated until use. The pancreatic juice was refrigerated at 4 °C if it was to be used within a few hours of collection, if not it was frozen at −20 °C until required. The pancreatic juice was used without purification. It contained 65 mg of protein/mL as measured by the Lowry assay.[46,47]

Competitive Hydrolysis of *RRR*-α-TOAc-d_0 and *SRR*-α-TOAc-d_3 Using Rat Bile and either Rat Pancreatic Juice or cBCE. Bile (400 μL), Tris buffer (0.3 mL), *dl*-DMPC (2 mM), and aqueous NaCl (150 mM, 2.5 mL) were added to a neat equimolar mixture containing 110 μM *RRR*-α-TOAc-d_0 and 110 μM *SRR*-α-TOAc-d_3. The resulting mixture was vortex stirred for 1 min and then incubated for 15 min at 37 °C, pH 7.7. Following the addition of either 10 μL of pancreatic juice or 20 μL of a nonstandardized solution of cBCE (1.1 mg protein/mL), 100 μL aliquots were withdrawn at 5 min intervals and were placed in tubes which contained 100 μL of 0.1 M sodium dodecyl sulfate (SDS), 200 μL EtOH, 200 μL heptane, and 50 μL of a 160 μM heptane solution of the *all-rac*-α-TOH-d_9 which was used as an internal standard for the GC/MS analysis of the *RRR*-α-TOH-d_0 and *SRR*-α-TOH-d_3. These aliquots were immediately vortex mixed, and the free tocopherols and tocopheryl acetates contained in the heptane layer were then separated by HPLC on Lichrosorb Si60 (5 μm) using a Varian 5000 instrument equipped with a Fluorochrome detector ($\lambda_{ex} = 295$ nm, $\lambda_{em} = 340$ nm). Analysis of the free tocopherols by GC/MS was performed on a Hewlett Packard 5890 gas chromatograph equipped with a 5970 Series mass selective detector as described previously.[38,50] In the experiments with the rat pancreatic juice and with the cBCE, the initial rates of hydrolysis of the acetates were considerably (ca. 5 times) greater and considerably lower, respectively, than the rates measured under the standard conditions which were maintained in most later experiments.

Preparation of Standard α-TOAc/*l*-DMPC Mixtures. Solutions containing 1.99 mM *RRR*-α-TOAc-d_6 in heptane, 2.26 mM *SRR*-α-TOAc-d_3 in heptane, and 32.0 mM *l*-DMPC[51] in CH_2Cl_2 were prepared. Aliquots of these stock solutions were mixed in screw-top vials, and the solvents were removed under a stream of nitrogen to give mixtures of *RRR*-α-TOAc-d_6/*l*-DMPC or *SRR*-α-TOAc-d_3/*l*-DMPC or equimolar *RRR*-α-TOAc-d_6/*SRR*-α-TOAc-d_3 with *l*-DMPC. A large number of

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(51) We have shown previously[35] that rates of the cholate-activated CE-catalyzed noncompetitive hydrolyses of *RRR*-α-TOAc and *SRR*-α-TOAc are the same when using *l*-DMPC as when using *dl*-DMPC. In the present work we show that this is also the case for the comparable competitive experiments *(vide infra)*.

these samples were prepared at one time and were stored at −20 °C until required.

An aliquot (1.6 mL) of a bile salt solution containing 100 mM sodium cholate, glycocholate, or taurocholate was added to the dry α-TOAc/*l*-DMPC mixture, and the sample was vigorously mixed for 1 min using a vortex mixer. Aliquots of Tris buffer (0.40 mL of 500 mM Tris made up from 300 mM Trizma HCl, 200 mM Trizma base, and 1.50 M NaCl, pH 8.0 at 37 °C) and distilled, deionized water (2.0 mL) were then added, and the solution was again briefly vortex mixed. An aliquot (1.5 or 3.0 mL) of this micellar solution was then equilibrated for 15 min at 37 °C prior to the addition of an aqueous solution of a CE preparation. In some additional experiments, the α-TOAc/*l*-DMPC/bile salt mixtures were subjected to 10 min sonication at ca. 20 °C prior to thermal equilibration. This step did not change the kinetic results but, to judge by the UV/visible absorption spectrum during the thermal equilibration period, a stable micellar solution was obtained immediately, rather than during the equilibration as was the case for nonsonicated samples.

The final concentrations of the components of the aqueous bile salt/buffer solutions were 100 μM *RRR*-α-TOAc-d_6 or 100 μM *SRR*-TOAc-d_3 or 200 μM *RRR*-α-TOAc-d_6 plus *SRR*-TOAc-d_3 (equimolar), 2.00 mM *l*-DMPC, 50 mM Tris buffer, and 40 mM bile salt.

Standard Conditions for the Noncompetitive and Competitive Hydrolysis of *RRR*- and *SRR*-α-TOAc Using CE Preparations and Bile Salts. Micellar solutions containing 100 μM *RRR*-α-TOAc-d_6 or *SRR*-α-TOAc-d_3 or an equimolar mixture of 100 μM *RRR*-α-TOAc-d_6 plus 100 μM *SRR*-α-TOAc-d_3, 2.00 mM *l*-DMPC, 50 mM Tris buffer, and 40 mM bile salt were dispensed as 3.0 mL samples into a specially designed, glass multiwell reactor in which the samples were maintained under an argon atmosphere. After incubation for 15 min at 37 °C (pH 8.0), aliquots of solutions of CE (cBCE, pBCE, cPCE, or pPCE) were added, these aliquots being of the size required to produce an apparent initial rate of formation of *SRR*-α-TOH-d_3 of ca. 5.0×10^{-8} M s^{-1} (as measured spectrophotometrically at 302 nm, *vide supra*) in the *SRR*-α-TOAc-d_3/cholate/*l*-DMPC system described above. The true initial rates of acetate hydrolysis were determined by GC/MS analysis for α-TOH (and α-TOAc) using 100 μL samples removed just prior to addition of the CE ($t = 0$) and at 2, 5, 10, 20, 40, 60, 90, 120, 150, 180, and 210 min. (Sample times were occasionally varied from those just quoted in order to improve the kinetic data.) These samples were "quenched" in 0.5 M SDS (0.2 mL) containing ca. 20 mg of sodium ascorbate (to protect the liberated α-TOH from reaction with adventitious oxygen). An ethanol solution (50 μL) containing α-TOAc-d_9 and α-TOH-d_9 (130 μM of each) was added so as to provide internal standards.[50] Following this, 500 μL of ethanol and 500 μL of heptane were added, and after brief vortex mixing, the heptane extracts were analyzed by GC/MS. Absolute yields of *RRR*-α-TOAc-d_6, *RRR*-α-TOH-d_6, *SRR*-α-TOAc-d_3, and *SRR*-α-TOH-d_3 were readily obtained. In this paper we report only the rates of formation of the free phenols since the sum, $([\alpha\text{-TOAc}] + [\alpha\text{-TOH}])_t$, was at all times equal to the initial concentration of α-TOAc, $[\alpha\text{-TOAc}]_{t=0}$, (after correction for the dilution introduced by our analytical procedure).

Effect of Reagent Concentrations on Relative and Absolute Rates of Acetate Hydrolysis. Noncompetitive and competitive cPCE-catalyzed hydrolyses of *RRR*-α-TOAc-d_6 and *SRR*-α-TOAc-d_3 at 37 °C in 50 mM Tris buffer and with 2.00 mM *l*-DMPC were carried out with cholate, glycocholate, and taurocholate exactly as described above *except* for the concentrations of the bile salts. These concentrations were 3, 5, 10, 20, 40, and 80 mM.

In a related series of experiments, cholate and taurocholate were held constant at 5.0 mM and cPCE-catalyzed reaction rates were measured for competitive experiments at *l*-DMPC concentrations of 2, 4, 6, 8, and 10 mM. Competitive experiments were also carried out with 5.0 mM cholate and *dl*-DMPC at concentrations of 2, 4, 6, 8, and 10 mM. In all these experiments, the solutions became cloudy when the concentration of DMPC exceeded that of the bile salt, i.e., at [DMPC] ≥ 6 mM.

In another limited series of experiments using cPCE, with 40 mM cholate, glycocholate, or taurocholate, the DMPC was completely eliminated. The rates of hydrolysis were then very slow but were generally somewhat accelerated by the addition of sodium oleate at concentrations of 0.5 and 2.0 mM. These experiments were carried out in the usual manner.

Dihydroxy Bile Salts. Some preliminary noncompetitive experiments were carried out to measure the extent of hydrolysis after 24 h of *RRR*- and *SRR*-α-TOAc using eight dihydroxy bile salts (three 3α,7α, three 3α,7β, and two 3α,12α) to activate/modulate cBCE under standard conditions (37 °C, 40 mM bile salt, 100 μM α-TOAc, pH 8.0, etc). These studies showed that only the three 3α,7α-dihydroxy bile salts had any significant ability to activate the CE. These three dihydroxy bile salts were therefore purified by recrystallization and subjected to study using the same reaction conditions as those employed with the trihydroxy bile salts.

Inhibition of α-TOAc Hydrolysis by α-TOH. Noncompetitive, bile salt (40 mM) stimulated, cBCE-catalyzed hydrolyses of *RRR*- and *SRR*-α-TOAc at concentrations of 40, 50, 63, 100, and 200 μM were carried out under standard experimental conditions in the absence and in the presence of 50 μM *RRR*- or *SRR*-α-TOH. Initial rates of hydrolysis were determined spectrophotometrically.[35]

Results

Molecular Weight of Reduced PCE and BCE. The molecular weight found in the present work for reduced PCE, viz., 58 kDa, is unexpectedly low, being lower, in fact, than nearly all the molecular weights which have been reported for any reduced CE.[52] Indeed, although our molecular weight for PCE lies within the range of reported values (45−83 kDa),[52] it does not actually agree with any previous measurement.[52] We did not pursue these PCE molecular weight "peculiarities" because they would appear to be irrelevant to the main thrust of our research and because crude PCE and pure PCE produced essentially identical diastereoselectivities in our experiments.

In contrast to PCE, our molecular weight for reduced BCE, viz., 65 kDa, is in good agreement with the literature.[52,56,57]

Competitive Hydrolysis of *RRR*-α-TOAc-d_6 and *SRR*-α-TOAc-d_3 in the Presence of Rat Bile. Although no attempt was made to obtain comparable rates of hydrolysis for the rat bile-modulated reactions catalyzed by pancreatic juice and by cBCE, the results are qualitatively the same in the only matter we consider to be important. This is that *RRR*-α-TOAc is hydrolyzed more rapidly than the *SRR* diastereomer (see Figure 1). This pair of experiments was critical since it demonstrated that the stereoselectivities of the carboxylic ester hydrolase(s) secreted by the rat and of cBCE were similar. Thus, and as mentioned in the Introduction, the failure of our *in vitro* cholate-modulated cBCE experiment[35] to "model" our *in vivo* experiments[38,39] could *not* be due to an intrinsic difference in the selectivities of the two esterases. Instead, it *had* to be related

(52) PCE: 80,[53] 45,[54] and 74 and 83 kDa.[55] BCE: 65,[56] and 67 and 72 kDa.[57] Rat CE: 55,[58] 65−69,[20,59] 67,[60] and 70 kDa.[15,31] Human CE: 100 kDa.[61−63]

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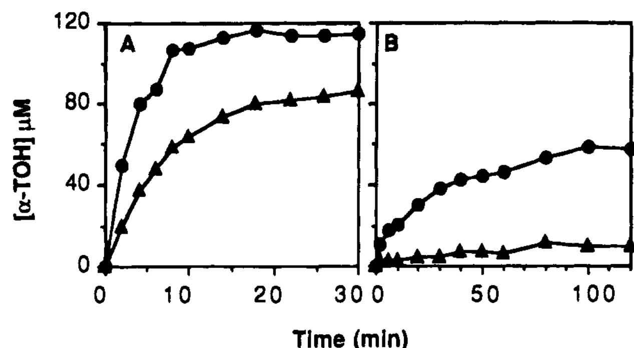

Figure 1. Formation of *RRR*-α-TOH-d_6 (●) and *SRR*-α-TOH-d_3 (▲) during the competitive hydrolyses of equal concentrations of *RRR*-α-TOAC-d_6 (110 μM) and *SRR*-α-TOAc-d_3 (110 μM) at 37 °C in the presence of rat bile (400 μL), Tris buffer (0.3 mL), *dl*-DMPC (2 mM), and aqueous NaCl (150 mM, 2.5 mL), pH 7.7. (A) Reaction initiated with 10 μL of rat pancreatic juice. (B) Reaction initiated with 20 μL of a solution of cBCE (1.1 mg of protein/mL).

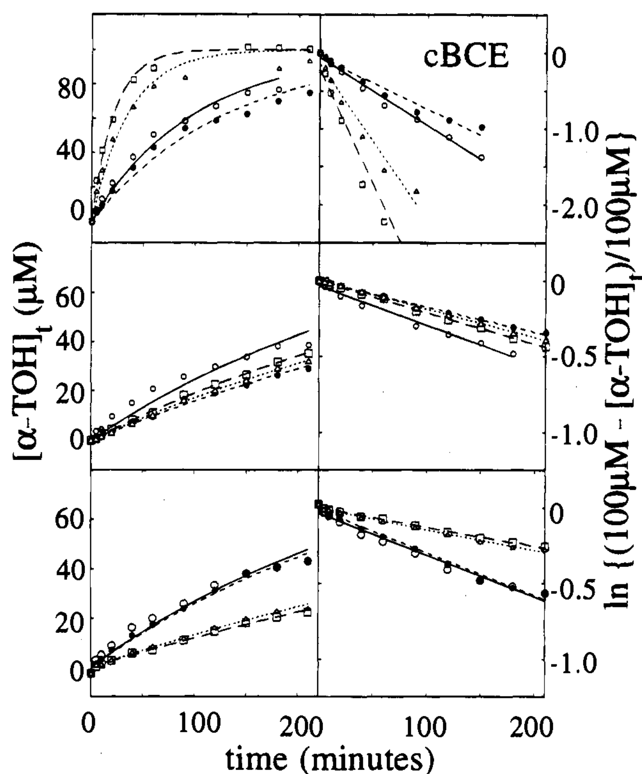

Figure 2. Formation of *RRR*-α-TOH-d_6 and *SRR*-α-TOH-d_3 during the noncompetitive, ○ and □, respectively, and competitive, ● and △, respectively, crude bovine cholesterol ester (cBCE)-catalyzed hydrolyses of 100 μM *RRR*-α-TOAc-d_6 or 100 μM *SRR*-α-TOAc-d_3 (noncompetitive) and 100 μM *RRR*-α-TOAc-d_6 plus 100 μM *SRR*-α-TOAc-d_3 (competitive) at 37 °C in the presence of 2 mM *l*-DMPC, 50 mM Tris buffer (pH 8.0), 40 mM of cholate (top two panels), glycocholate (middle two panels), and taurocholate (bottom two panels) dispersed in 3 mL of water.

to the nature of the bile salt used to activate the enzyme. The present work stems from this important discovery.

Noncompetitive and Competitive Hydrolyses of *RRR*-α-TOAc-d_6 and *SRR*-α-TOAc-d_3 Catalyzed by Four Different CE Preparations and Three Trihydroxy Bile Salts. Kinetic traces showing α-TOH formation as a function of time are shown for cBCE in Figure 2 and for cPCE in Figure 3 (left-hand panels). All of these traces were obtained under standard conditions in terms of temperature (37 °C), pH (8.0), and reagent concentrations (40 mM bile salt, 50 mM Tris buffer, 2 mM *l*-DMPC, and 100 μM α-TOAc in the noncompetitive experiments and 200 μM α-TOAc ([*RRR*] = [*SRR*] = 100 μM) in the competitive experiments with the CE concentration adjusted to (less than perfect) standard activity for the cholate-modulated hydrolysis of *SRR*-α-TOAc, see the Experimental Section).

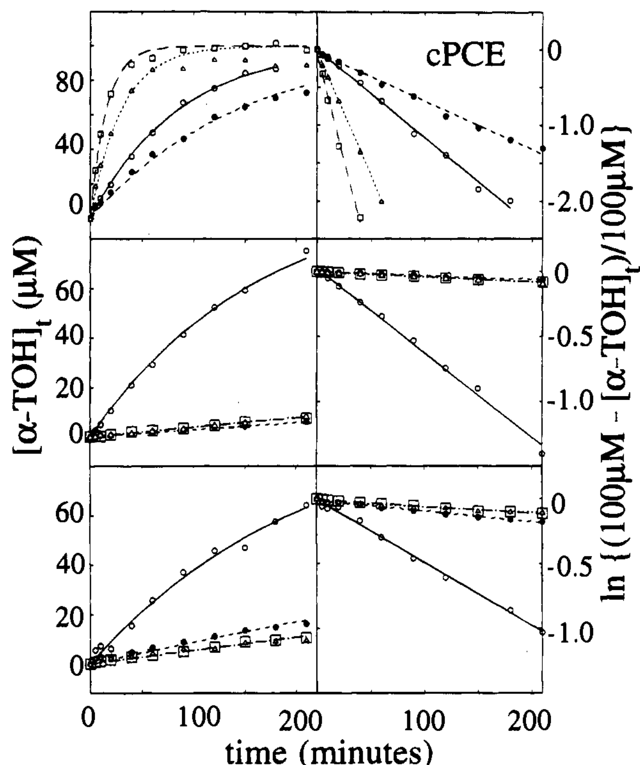

Figure 3. As for Figure 2 except for the use of crude porcine cholesterol esterase (cPCE).

The hydrolytic reactions illustrated in the left-hand panels of Figures 2 and 3 should follow pseudo-first-order kinetics, and hence, these curves can be linearized by converting them to semilogarithmic-type plots, i.e.

$$\ln\left\{\frac{[\alpha\text{-TOAc}]_{t=0} - [\alpha\text{-TOH}]_{t=t}}{[\alpha\text{-TOAc}]_{t=0}}\right\} \quad \text{vs time, } t$$

Since the initial concentration of acetate, $[\alpha\text{-TOAc}]_{t=0}$, was 100 μM in each experiment we can plot

$$\ln\{(100 - [\alpha\text{-TOH}]_{t=t})/100\} \quad \text{vs} \quad t\ (\text{s})$$

Plots of this type are shown in the right-hand panels of Figures 2 and 3. It can be seen that excellent straight lines are obtained even for reactions which reached fairly high conversions.[64] The slopes of these lines yield the initial rates of hydrolysis, V_i:

$$V_i = -(\text{slope})[\alpha\text{-TOAc}]_{t=0} \quad (\text{M s}^{-1})$$

$$V_i = -\frac{\ln\{(100 - [\alpha\text{-TOH}]_{t=t}/100)\}}{t} \times 10^{-4} \quad (\text{M s}^{-1})$$

Values of V_i calculated in this way for the experiments described above are given in Table 1.

(64) This probably implies that there is not much inhibition of hydrolysis by the α-TOH product under these conditions. We have previously demonstrated[35] that, in the cholate/cBCE/*l*- (or *dl*-) DMPC system, α-TOAc and α-TOH compete for the same binding site in the enzyme and that in this system the enzyme has a higher affinity for *RRR*-α-TOH than for *SRR*-α-TOH. Thus, product inhibition can occur under certain conditions. Inhibition by the phenol product has been further confirmed in the present work for the glycocholate- and taurocholate-stimulated reactions (*vide infra*).

Table 1. Effects of Trihydroxy Bile Salts and Cholesterol Esterase Preparation on the Initial Rates of Hydrolysis, V_i, of *RRR*-α-TOAc (100 μM) and *SRR*-α-TOAc (100 μM) in Noncompetitive and (Competitive) Experiments[a]

		$10^8 V_i$/(M s^{-1})[b]		V_i^{RRR}/V_i^{SRR} [c]
bile salt	CE	*RRR*	*SRR*	
cholate	cBCE	1.5 (1.5)[d]	7.1 (4.2)[d]	0.21 (0.36)[d]
	pBCE	1.6 (1.7)	7.5 (5.8)	0.21 (0.29)
	cPCE	1.9 (1.1)	9.2 (5.5)	0.21 (0.20)
	pPCE[e]	2.1 (1.3)	11 (6.0)	0.19 (0.22)
glycocholate	cBCE	0.43 (0.28)	0.35 (0.32)	1.2 (0.88)
	pBCE	0.42 (0.24)	0.24 (0.24)	1.8 (1.0)
	cPCE	1.1 (0.050)	0.062 (0.069)	18 (0.72)
	pPCE	1.0 (0.12)	0.13 (0.12)	7.7 (1.0)
taurocholate	cBCE	0.53 (0.53)	0.21 (0.25)	2.5 (2.1)
	pBCE	0.72 (0.55)	0.25 (0.24)	2.9 (2.3)
	cPCE	0.81 (0.15)	0.089 (0.074)	9.1 (2.0)
	pPCE[f]	0.86 (0.15)	0.069 (0.057)	12 (2.6)

[a] At 37 °C and pH 8.0 with 40 mM bile salt, 50 mM Tris buffer, 2 mM *l*-DMPC and 100 μM α-TOAc in the noncompetitive experiments and 200 μM α-TOAc ([*RRR*] = [*SRR*] = 100 μM) in the competitive experiments. Concentration of CE adjusted to (less than perfect) standard activity for the cholate modulated hydrolysis of *SRR*-α-TOAc (see text). [b]Rates calculated from semi-logarithmic plots (see text and right-hand panels in Figures 2 and 3). Individual rate measurements were reproducible to ±5%. [c]Errors for noncompetitive selectivity ratios are ±10% and for competitive selectivity ratios are ±5%. [d]Competitive experimental data are in parentheses. [e]Rates differ slightly from those given in ref. 34. [f]Rates differ significantly from those given in ref. 34. The previously reported rates[34] were inadvertently those which had been obtained with a badly contaminated sample of taurocholate which had not been recrystallized. We apologize for this error.

It is obvious from even the most cursory examination of Table 1 that, although the *direction* of chiral discrimination between *RRR*- and *SRR*-α-TOAc (in terms of their relative rates of hydrolysis, i.e., V_i^{RRR}/V_i^{SRR}) depends on the bile salt used and on whether the experiment was run in a noncompetitive or competitive manner, it is virtually independent of the purity of the BCE or PCE. However, the source of the enzyme (cow or pig) can influence noncompetitive experiments but it has little or no effect on discrimination in the competitive experiments.

Effect of Bile Salt Concentration. The effect of the concentration of the trihydroxy bile salts on the initial rates for the cPCE-catalyzed noncompetitive and competitive hydrolyses of *RRR*- and *SRR*-α-TOAc under otherwise standard experimental conditions (including 2.0 mM *l*-DMPC) is shown by the results listed in Table 2. There was no measurable reaction in the complete absence of bile salt, but reaction could be readily detected upon the addition of as little as 3 mM of these CE activators. In both the noncompetitive and competitive experiments the reaction rates do not change dramatically over a range of bile salt concentrations from 5 to 80 mM. Furthermore, the direction and (very roughly) the magnitude of the chiral discrimination induced by each bile salt are not greatly dependent on the bile salt concentration.

Effect of DMPC Concentration. Competitive cPCE-catalyzed hydrolyses were carried out using 5 mM cholate and 5 mM taurocholate with 2, 4, 6, 8, and 10 mM *l*-DMPC and using 5 mM cholate with the same concentrations of *dl*-DMPC.[65] Initial rates are given in Table 3. As was described in the Experimental Section, cloudy suspensions were formed when the concentration of DMPC reaches 6 mM. Under such conditions, the mixture forms an emulsion rather than a clear micellar solution. Although this causes the rates of hydrolysis to decline somewhat, the degree of diastereoselectivity would appear to be unaffected (see Table 3).

(65) Cholate and taurocholate were chosen for this study because they show the highest diastereoselectivity in competitive experiments with *RRR*- and *SRR*-α-TOAc (see Tables 1 and 2). The lower (5 mM) than normal (40 mM) concentration of these bile salts was chosen so as to be able to examine more easily what happened at high [DMPC]/[bile salt] ratios.

Table 2. Effects of Trihydroxy Bile Salt Concentrations on the Initial Rates of the cPCE-Catalyzed Hydrolysis, V_i, of *RRR*-α-TOAc (100 μM) and *SRR*-α-TOAc (100 μM) in Noncompetitive and (Competitive) Experiments[a]

	conc	$10^8 V_i$/(M s^{-1})[b]		V_i^{RRR}/V_i^{SRR} [c]
bile salt	(mM)	*RRR*	*SRR*	
	0	0.00 (0.00)[d]	0.00 (0.00)[d]	
cholate	3	0.082 (0.23)	1.2 (0.91)	0.068 (0.25)[d]
	5	0.41 (0.38)	3.2 (2.5)	0.13 (0.15)
	10	0.43 (0.34)	2.0 (1.5)	0.22 (0.23)
	20	0.57 (0.58)	5.7 (2.7)	0.10 (0.21)
	40	1.9 (1.1)	9.2 (5.5)	0.21 (0.20)
	80	1.9 (1.6)	5.6 (7.2)	0.34 (0.22)
glycocholate	3	0.34 (0.081)	0.11 (0.12)	3.1 (0.68)
	5	0.33 (0.046)	0.044 (0.042)	7.5 (1.1)
	10	0.60 (0.053)	0.077 (0.083)	7.8 (0.64)
	20	0.72 (0.048)	0.057 (0.055)	13 (0.87)
	40	1.1 (0.050)	0.062 (0.069)	18 (0.72)
	80	0.35 (0.041)	0.083 (0.067)	4.2 (0.61)
taurocholate	3	0.33 (0.10)	0.088 (0.064)	3.8 (1.6)
	5	0.36 (0.10)	0.087 (0.080)	4.1 (1.3)
	10	1.4 (0.22)	0.15 (0.14)	9.3 (1.6)
	20	2.8 (0.30)	0.18 (0.18)	16 (1.7)
	40	0.81 (0.15)	0.089 (0.074)	9.1 (2.0)
	80	0.71 (0.15)	0.12 (0.12)	5.9 (1.3)

[a] Except for the bile salt concentrations (when not equal to 40 mM), the experimental conditions were the same as those which generated the data given in Table 1. [b] See footnote *b* in Table 1. [c] See footnote *c* in Table 1. [d] See footnote *d* in Table 1.

Table 3. Effects of *l*- and *dl*-DMPC Concentration on the Initial Rates of the cPCE-Catalyzed Hydrolysis, V_i, of *RRR*-α-TOAc (100 μM) and *SRR*-α-TOAc (100 μM) in (Competitive) Experiments[a]

		$10^8 V_i$/(M s^{-1})[b]		V_i^{RRR}/V_i^{SRR} [c]
bile salt (5 mM)	[DMPC] (mM)	*RRR*	*SRR*	
cholate[d]	2	(0.38)[e]	(2.5)[e]	(0.15)[e]
	4	(0.41)	(2.8)	(0.15)
	6[f]	(0.35)	(3.1)	(0.11)
	8[f]	(0.31)	(2.1)	(0.15)
	10[f]	(0.072)	(0.59)	(0.12)
taurocholate[d]	2	(0.10)	(0.080)	(1.3)
	4	(0.14)	(0.066)	(2.1)
	6[f]	(0.12)	(0.064)	(1.9)
	8[f]	(0.081)	(0.040)	(2.0)
	10[g]			
cholate[h]	2	(0.27)	(1.5)	(0.18)
	4	(0.29)	(1.8)	(0.16)
	6[f]	(0.33)	(1.5)	(0.22)
	8[f]	(0.28)	(1.4)	(0.20)
	10[f]	(0.22)	(1.2)	(0.18)

[a] Except for the bile salt concentration and the DMPC concentration (when not equal to 2 mM), the experimental conditions were the same as those which generated the competitive data given in Table 1. [b] See footnote *b* in Table 1. [c] See footnote *c* in Table 1. [d] *l*-DMPC. [e] See footnote *d* in Table 1. [f] Solution slightly cloudy. [g] Rate not measurable due to instability of the micellar solution. [h] *dl*-DMPC.

Effect of Eliminating DMPC from the Reaction System. The possibility that what has been referred to above as the bile salt-modulated diastereoselectivity of CE was actually a bile salt/*l*-DMPC-modulated diastereoselectivity was explored by eliminating the DMPC and replacing it with sodium oleate (see Table 4). With no co-lipid to form mixed micelles with the bile salt, the rates of hydrolyses were very low. Nevertheless, in both the noncompetitive and the competitive experiments, the *directions* of the bile salt-induced chiral discrimination between *RRR*- and *SRR*-α-TOAc would generally appear to be the same as in the presence of DMPC. The addition of sodium oleate does not change the *direction* of the bile salt-induced

Table 4. Effects of No DMPC on the Initial Rates of the Trihydroxy Bile Salt-Activated cPCE-Catalyzed Hydrolysis, V_i, of *RRR*-α-TOAc (100 μM) and *SRR*-α-TOAc (100 μM) in Noncompetitive and (Competitive) Experiments[a]

bile salt (40 mM)	Na oleate (mM)	10^8V_i/(M s^{-1})[b] *RRR*	*SRR*	V_i^{RRR}/V_i^{SRR} [c]
cholate	0	0.066 (0.038)[d]	0.17 (0.081)[d]	0.39 (0.47)[d]
	0.5	0.16 (0.53)	2.5 (1.4)	0.064 (0.38)
	2	0.76 (0.73)	2.1 (1.4)	0.36 (0.52)
glycocholate	0	0.032 (0.010)	0.0088 (0.0046)	3.6 (2.2)
	0.5	0.051 (0.029)	0.028 (0.016)	1.8 (1.8)
	2	0.077 (0.026)	0.034 (0.017)	2.3 (1.5)
taurocholate	0	0.084 (0.019)	e	e
	0.5	0.068 (0.035)	0.031 (0.018)	2.2 (1.9)
	2	0.058 (0.025)	0.018 (0.013)	3.2 (1.9)

[a] At 37 °C under standard conditions except for the absence of DMPC. [b] See footnote *b* in Table 1. [c] See footnote *c* in Table 1. [d] See footnote *d* in Table 1. [e] Rate too low to be measured.

Table 5. Yields of α-TOH after 24 h for the Dihydroxy Bile Salt-Activated cBCE-Catalyzed Hydrolysis of *RRR*-α-TOAc (100 μM) and *SRR*-α-TOAc (100 μM) in Noncompetitive Experiments[a]

bile salt	positions hydroxylated	α-TOH (μM) *RRR*	*SRR*
none		0	0
chenodeoxycholate	3α,7α	8	21
glycochenodeoxycholate	3α,7α	84	36
taurochenodeoxycholate	3α,7α	30	25
ursodeoxycholate	3α,7β	0.6	0.3
glycoursodeoxycholate	3α,7β	3	1
tauroursodeoxycholate	3α,7β	1	1
deoxycholate	3α,12α	0	1
glycodeoxycholate	3α,12α	4	3

[a] Experimental conditions were the same as those later used to generate the data given in Table 1. Yields were measured spectrophotometrically.

chiral discrimination, but the rates of hydrolyses are generally increased, though not to the rates achieved with DMPC.[66] Since these experiments proved that the direction of the bile salt-induced chiral discrimination between *RRR*- and *SRR*-α-TOAc was not influenced by the use of DMPC as a co-lipid, the effect of oleate as a co-lipid was not investigated further.

Noncompetitive and Competitive Hydrolysis of *RRR*-α-TOAc-d_6 and *SRR*-α-TOAc-d_3 Catalyzed by cBCE or cPCE and Two Monohydroxy and Eight Dihydroxy Bile Salts. At a very early stage in the present work, we used the "old" spectrophotometric method to examine the effects of two mono- and eight dihydroxy bile salts on the cBCE-catalyzed noncompetitive hydrolysis of *RRR*- and *SRR*-α-TOAc. These bile salts were used as received. The two 3α-monohydroxy bile salts, lithocholate and taurolithocholate, failed to give clear micellar solutions suitable for spectrophotometric measurements of the rates of hydrolysis. They were not examined further.

The eight dihydroxy (deoxy) bile salts gave clear micellar solutions, but most of them were ineffective activators of the enzyme. In Table 5 are given the yields of α-TOH after 24 h at 37 °C for the reactions of 100 μM *RRR*-α-TOAc and 100 μM *SRR*-α-TOAc in a system comprising cBCE and bile salt (40 mM), *l*-DMPC (2 mM), and Tris buffer (50 mM) . It can be seen that only the three chenodeoxy (3α,7α-dihydroxy) bile salts were effective in activating the enzyme. A reasonably detailed study of the diastereoselectivities in the hydrolyses of *RRR*- and *SRR*-α-TOAc induced by the 3α,7α bile salts has been reported previously.[34] In brief, these three dihydroxy bile

(66) Oleate at concentrations greater than 2−4 mM has also been shown to inhibit the human CE-catalyzed hydrolysis of *all-rac*-α-TOAc and cholesterol oleate.[7]

Table 6. Effects of 3α,7α-Dihydroxy Bile Salts (40 mM) on the Initial Rates of the pPCE-Catalyzed Hydrolysis, V_i, of *RRR*-α-TOAc (100 μM) and *SRR*-α-TOAc in Noncompetitive and (Competitive) Experiments[a]

bile salt	10^8V_i/(M s^{-1})[b] *RRR*	*SRR*	V_i^{RRR}/V_i^{SRR} [c]
chenodeoxycholate	0.026 (0.022)[d]	0.040 (0.039)[d]	0.65 (0.56)[d]
glycochenodeoxycholate	0.77 (0.72)	0.072 (0.090)	11 (8.0)
taurochenodeoxycholate	0.036 (0.031)	0.029 (0.021)	1.2 (1.5)

[a] See footnote *a* in Table 1. [b] See footnote *b* in Table 1. [c] See footnote *c* in Table 1. [d] See footnote *d* in Table 1.

Table 7. Apparent Inhibition Constants, $K_{i(app)}$ for the α-TOH-Inhibited, Bile Salt-Modulated, cBCE-Catalyzed Hydrolyses of *RRR* and *SRR*-α-TOAc[a]

bile salt	substrate	inhibitor ($10^5K_{i(app)}$/(M) *RRR*-α-TOH	*SRR*-α-TOH
cholate	*RRR*-α-TOAc	6.4[b]	13.4[b]
	SRR-α-TOAc	7.2[b]	17.5[b]
glycocholate	*RRR*-α-TOAc	7.7	6.6
	SRR-α-TOAc	7.1	7.2
taurocholate	*RRR*-α-TOAc	28	10
	SRR-α-TOAc	c	c

[a] At 37 °C with 40 mM bile salt, 50 mM Tris buffer (pH 8), 1.7 mM *l*-DMPC, 40−200 μM α-TOAc, α-TOH (0 or 50 μM), and cBCE (4 mg/mL). [b] Data from reference 35. [c] Reaction was too slow to monitor spectrophotometrically.

salts have only a negligible ability to activate the BCE-catalyzed hydrolysis of either of the diastereomeric acetates during 3 h of incubation under standard conditions.[67] However, they do activate PCE (see Table 6), with chenodeoxycholate and taurochenodeoxycholate both showing a lower activity than the corresponding trihydroxy bile salts (cf. Table 1). However, glycochenodeoxycholate is about as effective an activator of PCE as glycocholate in the noncompetitive experiments and it is even more effective than glycocholate in the competitive experiment insofar as the hydrolysis of *RRR*-α-TOAc is concerned (cf. Tables 1 and 6). Indeed, this particular dihydroxy bile salt shows the highest diastereoselectivity that we have encountered in any competitive experiment, viz., V_i^{RRR}/V_i^{SRR} = 8.0.

Product Inhibition. By following the procedure previously employed,[35] the reciprocal of the (spectrophotometrically measured) initial rates of the glycocholate- and taurocholate-modulated, cBCE-catalyzed, (noncompetitive) hydrolyses of *RRR*- and *SRR*-α-TOAc were plotted against the reciprocal of the α-TOAc concentration (range 40−200 μM) in the absence and in the presence of 50 μM *RRR*- and *SRR*-α-TOH. As we have shown for the cholate-stimulated reactions, such plots yield straight lines which exhibit a common intercept at [*RRR*- or *SRR*-α-TOAc] = 0.[35] From the slopes of these lines, apparent inhibition constants, $K_{i(app)}$, can be calculated. These are given in Table 7.

Discussion

The origins of our almost inadvertent venture into enzyme chemistry were outlined in the Introduction. An intriguing failure to reproduce an *in vivo* experimental result *in vitro* has led, after a great deal of work, to the unequivocal confirmation of a most unusual and exciting discovery,[33] to wit: the diastereoselectivity for the CE-catalyzed hydrolysis of *RRR*- vs

(67) We attribute the early positive results with BCE to impurities (probably trihydroxy bile salts) in the commercial 3α,7α-dihydroxy bile salts which were employed in this early study.

SRR-α-TOAc can be modulated by the choice of bile salt used to activate the enzyme. More recently, we have examined the bile salt-modulated, CE-catalyzed hydrolyses of three acetates structurally analogous to α-TOAc but in which the phytyl "tail" of vitamin E (with its two chiral centers at positions 4′ and 8′) had been replaced by an *n*-alkyl group having 11, 13, or 15 carbon atoms.[68] For all three of these esters, each of the three trihydroxy bile salts induce a 2*R* vs 2*S* *enantioselectivity* for the hydrolyses which is in the same direction and has a similar magnitude to the diastereoselectivities observed in the corresponding hydrolyses of *RRR* vs *SRR*-α-TOAc.[68]

The discovery of bile salt-*modulated diastereoselectivity* and *enantioselectivity* is unusual and exciting because a major characteristic of catalysis by enzymes is their stereoselectivity.[69] Of course, the chiral selectivity of an enzyme can be modified if the precise structure of the active site is changed. This has sometimes been achieved by the heroic procedure of "protein engineering"[70] and sometimes by the simpler expedient of unfolding and then refolding the enzyme,[71] or even more simply by changing the reaction medium.[72] In the present instance, we simply change—in what would appear to be a very minor way (see Chart 1)—the structure of the surface active compound which is used to solubilize the substrate and activate the enzyme.

All three of the 3α,7α,12α-trihydroxy bile salts activate CE from two species of animals (cow and pig) and of various degrees of purity and thus allow the hydrolysis of both α-TOAc diastereomers to occur. The rates of hydrolyses in both the noncompetitive and competitive experiments are much lower when the bile acids are used without a co-lipid or with added oleate than when *l*- or *dl*-DMPC is present together with the bile salt (cf. Tables 1−4). However, the presence or absence of a co-lipid and its quantity relative to the (obligatory) bile salt only affect the reaction rates, they do not produce a significant difference in the *direction* of the diastereoselectivity for *RRR*- vs *SRR*-α-TOAc induced by the bile salt in either the noncompetitive or the competitive experiments.[73] All of this suggests (though it certainly does not prove) that the detailed structure of the bile salt micelle does not determine the stereoselectivity. Rather, the bile salts would appear to modulate the diastereoselectivity (and enantioselectivity) of CE by a "direct" effect on the protein which may involve a "refolding" of the enzyme, with such a "refolding" producing a change in the shape of the active site. In this connection, Wang and Hartsuck[4] have stated that, although the "mechanism of bile salt activation of BAL [i.e., CE, see the Introduction] is not understood in structural terms [, it] is reasonable to suggest that bile salt causes conformational change in BAL to provide active site access for the bulky substrate molecule." The actual hydrolysis undoubtedly occurs via a typical serine hydrolase mechanism.[18,74] Experimental work on human CE[10] and on the bile salt-stimulated human milk lipase[75] has led to the conclusion that there are two bile salt binding sites on each of these enzymes. While there is some disagreement about the class of bile salts (pattern of hydroxylation) which bind to each site, it would seem possible that one site activates the enzyme and controls its stereoselectivity while the second site encourages the binding of the CE to the surface of micellized lipids. The interfacial binding of CE to the bile salt/lipid mixed micelle is, presumably, mainly or entirely due to the electrostatic attraction between the negatively charged bile salts at the micellar surface and a positively charged CE surface (probably provided by arginine residues).[76]

We suggest that the differences between the noncompetitive and competitive diastereoselectivities are due to the fact that competitive inhibition of the enzyme by one diastereomeric acetate or its phenolic product can, in the competitive experiment, retard hydrolysis of the other acetate diastereomer. Inhibition of the cBCE-catalyzed hydrolysis by the product phenols is not very strong (cf. Table 7) and, as would be expected for competitive inhibition, the $K_{i(app)}$ values depend, within our experimental accuracy, only on the stereochemistry of the inhibitor (α-TOH), not on the stereochemistry of the substrate (α-TOAc). In the presence of cholate (which preferentially induces the BCE-catalyzed hydrolyses of *SRR*-α-TOAc in both the noncompetitive and the competitive experiments), inhibition by *RRR*-α-TOH is stronger than by *SRR*-α-TOH; i.e., $K_{i(app)}$ for inhibition by *RRR*-α-TOH of the hydrolyses of both *RRR*- and *SRR*-α-TOAc is smaller than $K_{i(app)}$ for inhibition by *SRR*-α-TOH. The cholate-modulated hydrolyses are all faster than the glycocholate- and taurocholate-modulated reactions (cf. Tables 1 and 2), and it is probably for this reason that competitive inhibition is unimportant and that the diastereoselectivities for noncompetitive and competitive experiments are essentially equal. In the presence of glycocholate (which, to a small extent, preferentially induces the BCE-catalyzed hydrolyses *RRR*-α-TOAc in noncompetitive experiments but shows no preference in the competitive experiments), *RRR*- and *SRR*-α-TOH are equally effective inhibitors, and with taurocholate (which preferentially induces the BCE-catalyzed hydrolyses of *RRR*-α-TOAc in both noncompetitive and competitive experiments), inhibition by *SRR*-α-TOH is stronger than by *RRR*-α-TOH. It is worth noting that only with cholate do the BCE- and PCE-catalyzed hydrolyses of *RRR*- and *SRR*-α-TOAc occur at very roughly similar rates in the noncompetitive and competitive experiments (cf. Tables 1 and 2). With glycocholate and taurocholate, this is also true for the BCE-catalyzed reactions but it is not true for the PCE-catalyzed

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(73) From the data in Tables 1−4 the average values of V_i^{RRR}/V_i^{SRR} for the noncompetitive and competitive (in parentheses) BCE-catalyzed hydrolyses can be calculated to be 0.21 (0.33) for cholate, 1.5 (0.94) for glycocholate, and 2.7 (2.2) for taurocholate, and for the PCE-catalyzed hydrolyses, 0.21 (0.21) for cholate, 7.9 (1.1) for glycocholate, and 7.5 (1.8) for taurocholate.

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hydrolysis. Instead, there is a drastic decline in the rate of the PCE-catalyzed hydrolysis of *RRR*-α-TOAc in the competitive experiment but not in the rate of hydrolysis of *SRR*-α-TOAc. This leads to much lower diastereoselectivities in the competitive experiments than the selectivities calculated from the noncompetitive rate data (cf. Tables 1 and 2). Although glycochenodeoxycholate does not activate BCE, it is very effective with PCE (cf. Table 6). This bile salt/CE combination induces the hydrolyses of the two diastereomeric esters at essentially the same rate in the competitive experiment as in the noncompetitive experiments. As a consequence, this bile salt/CE combination produces a uniquely high diastereoselectivity in the competitive experiment.

The competitive inhibition by product experiments (Table 7) indicate that the higher the affinity between the enzyme and one diastereomeric product phenol the lower the rate at which that diastereomer of the substrate acetate will be hydrolyzed. This appears quite reasonable for the noncompetitive reactions since it *could* mean that escape of the product from the enzyme's active site is the rate-limiting step in the hydrolysis. In a competitive experiment, the same phenomenon of slower reaction for one diastereomer because of the slow escape of its product from the active site would be bound to lead to a less dramatic diastereoselectivity for hydrolysis than in the noncompetitive experiments. This is, in fact, just what is observed in the glycocholate- and taurocholate-modulated reactions (cf. Tables 1 and 2). There is little difference in diastereoselectivity for the relatively rapid cholate-modulated hydrolyses. We therefore tentatively suggest that the observed diastereoselectivities for hydrolyses in noncompetitive *and* competitive experiments are controlled partly or entirely by differences in the rates of escape of the diastereomeric phenol products.

Summary. We have discovered a quite remarkable bile salt modulation of the diastereoselectivity of the CE-catalyzed hydrolyses of *RRR*- and *SRR*-α-TOAc. In competitive experiments (which are, of course, far more significant than the noncompetitive experiments if the bile salt-modulating effect on CE-catalyzed reactions is to be exploited in organic syntheses), the diastereoselectivity, V_i^{RRR}/V_i^{SRR}, varies from a high of 8.0 for the glycochenodeoxycholate/PCE couple to a low of 0.21 for the cholate/PCE couple. Such a dramatic 40-fold change in an enzyme's chiral selectivity is without precedent—and, as yet, explanation!

What is truly remarkable is the fact that the chiral carbon atom is separated by six bonds from the bond which is broken. This chiral center must exert its effect either prior to, or during, the rate-limiting step in the overall hydrolysis, this step being likely to be the formation of the ester/CE tetrahedral intermediate. The fact that it is the bile salt alone which determines the direction and magnitude of the *RRR* vs *SRR*-α-TOAc diastereoselectivity (and not the nature of the co-lipid nor the bile salt/co-lipid ratio) implies that the selectivity is probably not determined by the precise surface structure of the micelle on which the enzyme "sits and acts".

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